NF-κB Mediates Mitogen-Activated Protein Kinase Pathway-Dependent iNOS Expression in Human Melanoma

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Tumor expression of inducible nitric oxide synthase (iNOS) predicts poor outcomes for melanoma patients. We have reported the regulation of melanoma iNOS by the mitogen-activated protein kinase (MAPK) pathway. In this study, we test the hypothesis that NF-κB mediates this regulation. Western blotting of melanoma cell lysates confirmed the constitutive expression of iNOS. Western blot detected baseline levels of activated nuclear extracellular signal-regulated kinase and NF-κB. Indirect immunofluorescence confirmed the presence of NF-κB p50 and p65 in melanoma cell nuclei, with p50 being more prevalent. Electrophoretic mobility shift assay demonstrated baseline NF-κB activity, the findings confirmed by supershift analysis. Treatment of melanoma cells with the MEK inhibitor U0126 decreased NF-κB binding to its DNA recognition sequence, implicating the MAPK pathway in NF-κB activation. Two specific NF-κB inhibitors suppressed iNOS expression, demonstrating regulation of iNOS by NF-κB. Several experiments indicated the presence of p50 homodimers, which lack a transactivation domain and rely on the transcriptional coactivator Bcl-3 to carry out this function. Bcl-3 was detected in melanoma cells and co-immunoprecipitated with p50. These data suggest that the constitutively activated melanoma MAPK pathway stimulates activation of NF-κB hetero- and homodimers, which, in turn, drive iNOS expression and support melanoma tumorigenesis.

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INTRODUCTION

Malignant melanoma is currently the most virulent form of skin cancer, with incidence and mortality rates steadily rising in the US population (Jemal *et al.*, 2007). Although much improvement has been achieved regarding the treatment of early-stage disease, many patients die each year from melanoma metastases as tumor cells become increasingly resistant to chemotherapy. As such, the elucidation of pathways supporting metastatic cell growth is needed to develop specific targeted therapies for advanced disease.

The p44/42 mitogen-activated protein kinase (MAPK) pathway is arguably the most critical signaling cascade supporting the uncontrolled growth of melanoma cells (Smalley, 2003). In normal cells, the MAPK pathway is cytokine inducible. Signal induced by ligand binding is transported to a series of protein kinases that eventually culminate in the phosphorylation and activation of the extracellular signal-regulated kinase (ERK). Activated ERK translocates to the nucleus and initiates the transcription of a variety of growth-related genes. In melanoma cells, the MAPK pathway is constitutively active, a condition attributed in part to somatic mutations of *NRAS* and *BRAF*, which occur in the majority of cases (Goydos *et al.*, 2005). The aberrant activation of this pathway confers a protective benefit to melanoma cells by driving their proliferation and survival. The critical nature of the MAPK pathway has generated interest in melanoma-specific targets downstream of ERK that may participate in these oncogenic processes.

One such target of particular interest to our laboratory is inducible nitric oxide synthase (iNOS). The catalytic product of iNOS is nitric oxide (NO), a diatomic free radical that mediates processes such as neurotransmission, vasodilation, and host defense in normal cells. NO has been shown to contribute to the pathogenesis of a variety of cancers, as well. (Thomsen *et al.*, 1994; Cobbs *et al.*, 1995). Specifically, in melanoma, iNOS-generated NO has been shown to protect tumor cells from apoptosis (Salvucci *et al.*, 2001; Tang and Grimm, 2004). We have recently reported that the MAPK pathway drives constitutive iNOS expression in melanoma cells (Ellerhorst *et al.*, 2006). Furthermore, we have published data demonstrating an association of tumor iNOS expression with poor prognosis in patients diagnosed with stage III melanoma (Ekmekcioglu *et al.*, 2006).

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Abbreviations: EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PBS, phosphate-buffered saline; pERK, phosphorylated ERK

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The findings described above have prompted us to explore the molecular links between melanoma ERK and iNOS, to further clarify this tumor-promoting pathway. A particularly promising candidate protein is the transcription factor NF-κB, the activation of which is a hallmark of many cancers, including melanoma. (Wang *et al.*, 1999; Huang *et al.*, 2000; Dhawan and Richmond, 2002; Tian *et al.*, 2006). NF-κB consists of a family of structurally related proteins, including Rel A (p65), Rel B, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). In quiescent cells, NF-κB exists as cytoplasmic hetero- or homodimers associated with an inhibitory protein belonging to the IκB family. Upon appropriate cytokine stimulation, IκB kinase (IKK) induces the phosphorylation of IκB, promoting its subsequent ubiquitination and degradation in the proteasome (Baldwin, 1996). This process allows the translocation of NF-κB to the nucleus where it binds to target DNA elements and induces transcription of various genes involved in inflammation and survival, including *iNOS* (Taylor *et al.*, 1998). Evidence for a role of MAPK in NF-κB activation has been reported in several nonmelanoma systems, as well as in the HS294T human melanoma cell line (Wang *et al.*, 1999; Dhawan and Richmond, 2002; Kurland *et al.*, 2003; Jiang *et al.*, 2004).

We have thus hypothesized that NF-κB serves as the regulatory link between the MAPK pathway and iNOS expression in human melanoma cells. In this study, we explore this proposed sequence of molecular signaling events and show that, indeed, the constitutively activated melanoma MAPK pathway drives NF-κB activation, which, in turn induces iNOS expression.

RESULTS

Human melanoma cells constitutively express activated ERK, activated NF-κB, and iNOS

Initial experiments were performed to demonstrate the presence in melanoma cells of the three proteins included in our proposed pathway: ERK, NF-κB, and iNOS. The human melanoma cell lines WM35, WM793, MeWo, and HS294T, as well as primary melanocytes (FMC7C and FMC14C), were used in these experiments. WM793 and WM35 carry *BRAF* T1799A mutations, whereas MeWo, HS294T, and both melanocyte lines are *BRAF* wild type (Figure S1). None of these lines carry *NRAS* mutations. Nuclear extracts prepared from these cells under basal growth conditions contained both NF-κB p50 and p65, and phosphorylated ERK (pERK), indicating the presence of these proteins in the activated form (Figure 1a). Nuclear expression varied from one cell line to the next, but tended to be higher in the melanoma cells when compared to the melanocytes (Figure 1c). A western blot of whole-cell lysates from the same cells revealed the constitutive presence of iNOS protein and demonstrated the same pattern of higher levels in the melanoma cells (Figures 1b and c). An unanticipated observation was the presence of iNOS protein in the nuclear extracts of the melanoma cell lines and melanocytes (Figure 1b), a finding that has not been previously reported.

Indirect immunofluorescence studies were next carried out to confirm the nuclear localization of NF-κB in these cells. In keeping with the immunoblotting data, constitutive translocation of NF-κB p50 and p65 to the nuclei of melanoma cells was demonstrated (Figure 2). Because nuclear NF-κB can exist as p50/p65 heterodimers or p50 homodimers, the prevalence of each of these NF-κB subunits was examined in the immunofluorescently stained cells. Among the melanoma cell lines, there was a significant difference in the number of cells expressing nuclear p50 and p65, with some cells expressing only p50 (Figure 3). This finding was not observed in the melanocytes (FMC7C). These data suggest that melanoma cells utilize both hetero- and homodimeric NF-κB forms for transcriptional activation, and that the p50 homodimer activity may be tumor specific.

The melanoma MAPK pathway regulates NF-κB

The first segment of the proposed MAPK/NF-κB/iNOS pathway involves the regulation of melanoma NF-κB by MAPK. An electrophoretic mobility shift assay (EMSA), carried out with nuclear extracts from WM793 cells at baseline, revealed NF-κB binding to its consensus oligonucleotide DNA target sequence (Figure 4a). Supershift experiments with antibodies to NF-κB p50 and p65 revealed even greater retardation of the DNA/protein/antibody complexes. The antibody to NF-κB p50 produced a more complete supershift than antibody to the p65 subunit, consistent with the retardation of both p50 homodimers and p50/p65 heterodimers by the anti-p50 antibody, whereas the p65 antibody recognizes only the heterodimers, leaving the homodimers unaffected (Figure 4b).

Additional EMSA experiments were performed using WM793 cells to assess the effects of the MEK inhibitor U0126 on NF-κB nuclear-binding activity to test the hypothesis that NF-κB is regulated by the melanoma MAPK pathway. These experiments revealed diminished NF-κB binding as early as 2 minutes, with nearly complete abrogation of activity after 5 minutes of exposure to U0126 (Figure 4c). Experiments repeated with MeWo cells yielded similar results (data not shown). These data convincingly support the activation of NF-κB by the MAPK pathway in melanoma cells.

NF-κB regulates iNOS expression in melanoma cells

The second segment of the proposed pathway involves the induction of melanoma iNOS expression by NF-κB. To examine this regulatory event, melanoma cells were treated with each of two inhibitors that target different aspects of NF-κB activation. The first, NF-κB activation inhibitor, interferes with the transcriptional ability of NF-κB, whereas the second inhibitor, IKK-2 inhibitor V, selectively blocks IκBα phosphorylation, preventing the translocation of NF-κB to the nucleus.

Figure 5a shows the inhibition of iNOS protein expression in MeWo cells over the course of a 48-hours incubation with NF-κB activation inhibitor. Similar findings are demonstrated in WM793 cells treated with IKK-2 inhibitor V (Figure 5b); in this experiment, the decline in iNOS levels is preceded by a reduction in phosphorylation of IκBα. With both inhibitors, the stable, dimeric iNOS functional unit persists for the first

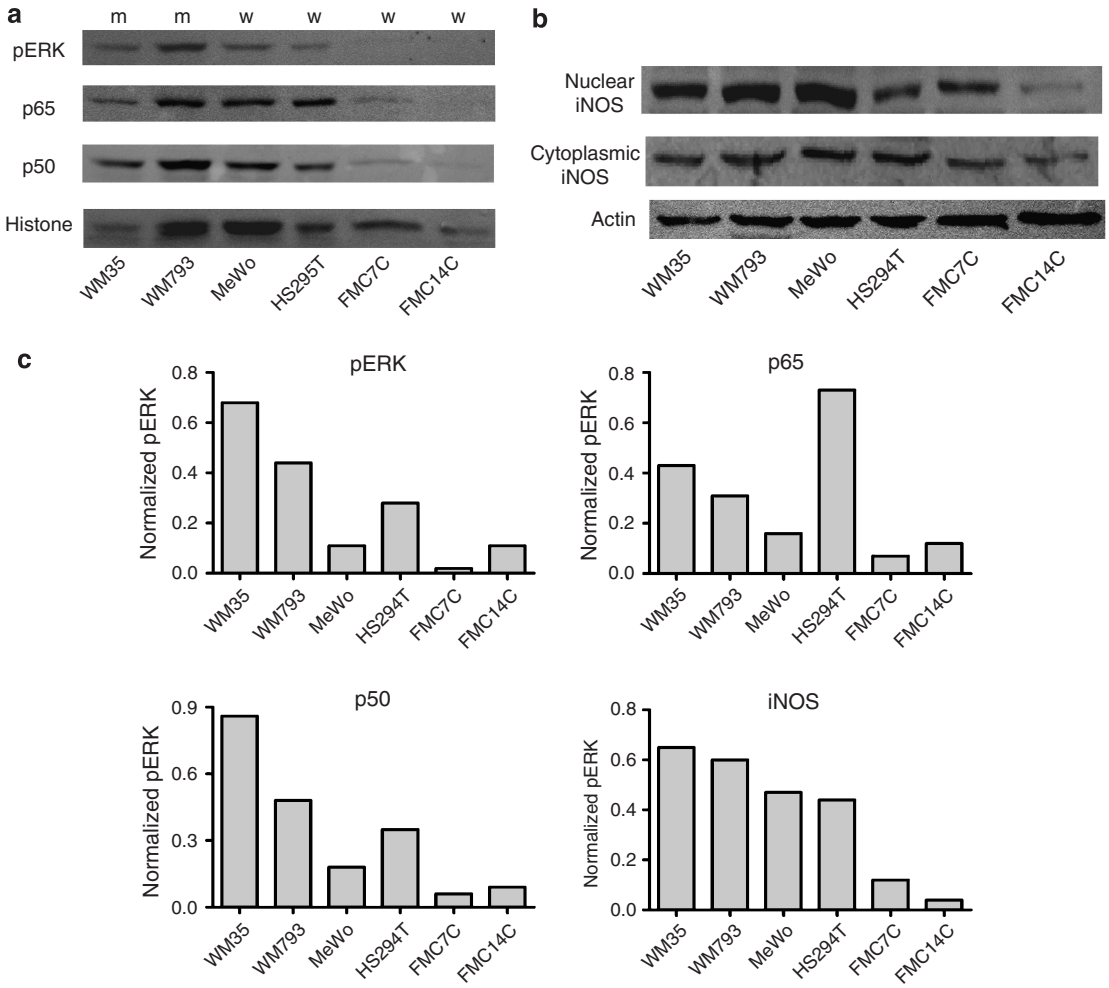

Figure 1. Expression of activated NF-κB, activated ERK, and iNOS in melanoma cell lines and melanocytes. (**a**) Western blotting of nuclear extracts confirms the nuclear localization of phosphorylated extracellular signal-regulated kinase (pERK), and NF-κB p50 and p65 in these cell lines. Histone serves as a loading control. (**b**) Western blot of whole-cell lysates demonstrates the constitutive presence of inducible nitric oxide synthase (iNOS) in melanoma cells and melanocytes. iNOS is present in nuclear extracts as well. (**c**) Normalization of the immunoblotting results in (**a** and **b**) to the histone or actin bands is shown, to control for differences in loading and transfer. The iNOS chart refers to cytoplasmic levels. m, *BRAF* mutant cell lines; w, *BRAF* wild-type cell lines.

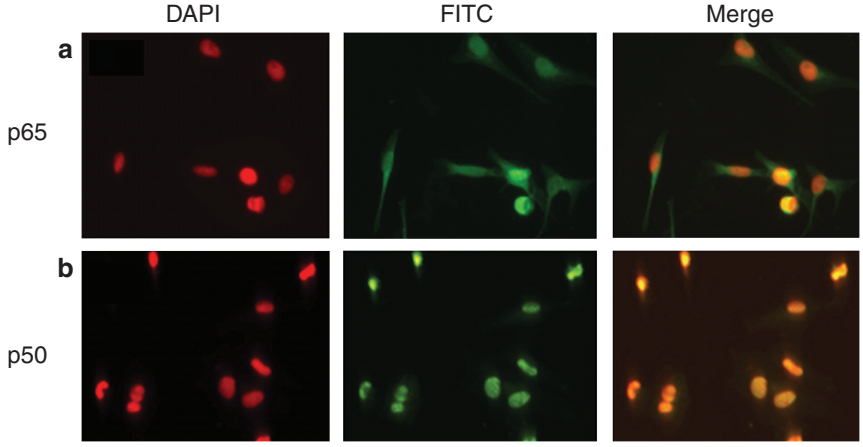

Figure 2. Nuclear localization of NF-κB p50 and p65. Immunofluorescent staining reveals the presence of the NF-κB subunits in (**a**) MeWo and (**b**) HS294T cells.

12 hours, but is markedly diminished thereafter. Similar findings were seen with WM35 cells (data not shown). These results are consistent with the hypothesis that NF-κB in human melanoma induces iNOS expression.

Melanoma cells express the transcriptional coactivator Bcl-3 that complexes with NF-κB p50

Data from several of the experiments described above indicated the presence of both NF-κB p50 homodimers and p50/p65 heterodimers in the nuclei of melanoma cells. The p50 homodimer binds DNA *in vitro*, but lacks a transcriptional activation domain and, consequently, initiates transcription weakly, if at all. In fact, this NF-κB homodimer appears to function as transcriptional inhibitor in some cell types by competing with p50/p65 for promoter binding (Kang *et al.*, 1992; Plaksin *et al.*, 1993). Countering this inhibitory effect in a variety of transformed cell types is the oncoprotein Bcl-3, which binds NF-κB p50 homodimers and robustly coactivates transcription (Fujita *et al.*, 1993). Experiments were thus conducted to explore a potential role of Bcl-3 in melanoma NF-κB p50 transcriptional coactivation. Western blots of whole-cell lysates and nuclear extracts from melanoma cells revealed the presence of the Bcl-3 protein in both the cytoplasmic and nuclear compartments (Figure 6a). Notably, Bcl-3 was virtually undetectable in melanocytes. Subsequently, the association of Bcl-3 and NF-κB p50 in melanoma cells was examined. Proteins from whole-cell

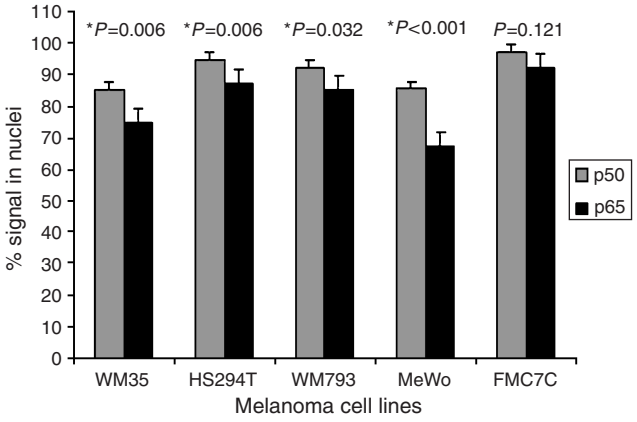

Figure 3. Comparison of the prevalences of NF-κB p50 and p65 signals in the nuclei of melanoma cells and melanocytes. A significantly higher number of melanoma cells express p50 relative to p65. Error bars represent the mean ± s.d. *P-values were found to be statistically significant by χ^2-analysis.

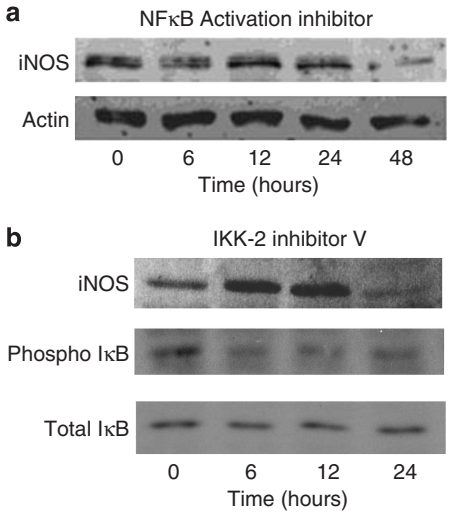

Figure 5. NF-κB regulates iNOS expression in melanoma cells. MeWo cells (**a**) were treated with the NF-κB activation inhibitor (10 μM) and WM793 cells (**b**) with IκB kinase (IKK)-2 inhibitor V (1 μM) over a time course of up to 48 hours. Western blotting was performed for inducible nitric oxide synthase (iNOS) protein and, in the case of IKK-2 Inhibitor V, for total and phosphorylated IκBα.

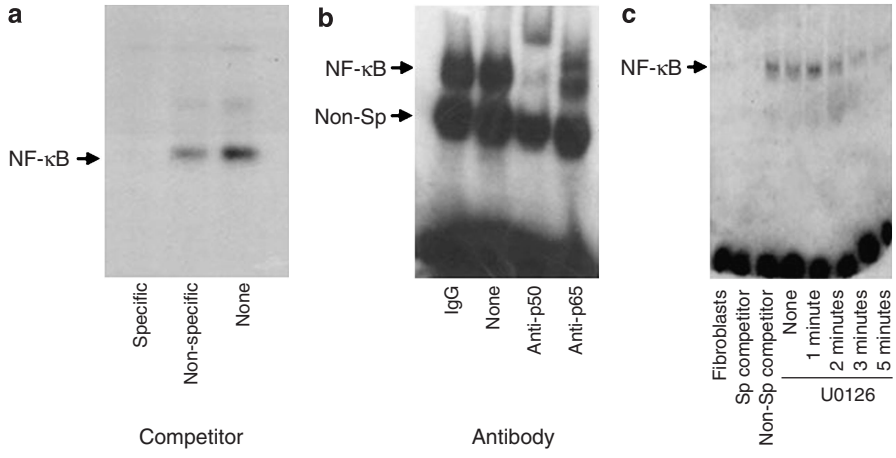

Figure 4. Melanoma NF-κB is regulated by the MAPK pathway. (**a**) Electrophoretic mobility shift assay (EMSA) was carried out with ^{32}P-labeled NF-κB target oligonucleotide and 4 μg of nuclear protein from unstimulated WM793 cells. Binding experiments were performed in the presence of a specific competitor (unlabeled NF-κB oligonucleotide), a nonspecific competitor (SP1 target oligonucleotide), and in the absence of competitor. Results demonstrate NF-κB-binding activity in these lysates. (**b**) Supershift experiments were conducted to confirm the specificity of NF-κB binding. Lysates (4 μg) were incubated with 1 μg of antibody to p50 or p65, followed by EMSA analysis. (**c**) Nuclear extracts of WM793 cells treated with 10 μm U0126 were prepared and examined for NF-κB activity. Reversal of NF-κB binding is observed. Sp, specific.

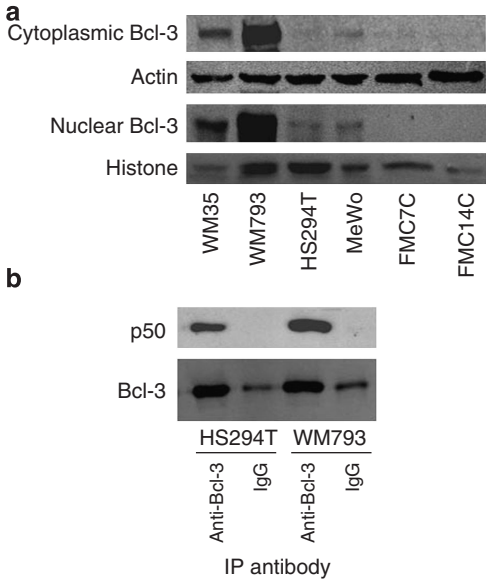

Figure 6. Melanoma cells express Bcl-3/p50 complexes. (**a**) Whole-cell lysates and nuclear extracts from melanoma cells and melanocytes were used to carry out western blotting for Bcl-3. (**b**) Whole-cell lysates from melanoma cells were used for immunoprecipitation with anti-Bcl-3 antibody followed by western blotting with anti-p50.

lysates were immunoprecipitated with rabbit IgG or anti-Bcl-3 antibody, followed by western blotting with antibody to p50. Figure 6b demonstrates the presence of NF-κB p50 in the Bcl-3 immunoprecipitates, indicating the presence of Bcl-3/p50 complexes in melanoma cells, with the potential for transcriptional activity.

DISCUSSION

The Ras/Raf/MEK/ERK pathway is a major signaling pathway involved in tumor cell growth and survival. As human melanoma is driven by constitutive activation of this pathway, elucidation of downstream effector molecules is critical to the understanding of melanoma pathobiology. We have previously reported the positive regulation of melanoma iNOS expression by the MAPK pathway and the profoundly negative clinical implications of tumor iNOS expression in this malignancy (Ellerhorst *et al.*, 2006; Ekmekcioglu *et al.*, 2006). We now show that NF-κB mediates this regulatory event. Previous work in the literature has described the activation of NF-κB by the MAPK pathway in the HS294T human melanoma cell line (Dhawan and Richmond, 2002). Our data now confirm and extend this finding, as we demonstrate that the inhibition of MAPK signaling with the MEK inhibitor U0126 is accompanied by diminished nuclear NF-κB DNA binding. Furthermore, this is this first study to show that NF-κB regulates expression of iNOS in melanoma cells. Notably, the MAPK/NF-κB/iNOS pathway was demonstrated in both *BRAF* mutant and wild-type cells, suggesting that the source of MAPK activation does not alter these downstream events.

An unexpected finding was the presence of iNOS in the nuclei of cultured melanoma cells and melanocytes. Although nuclear iNOS has been reported in neutrophils and adipocytes of rat origin (Giordano *et al.*, 2002; Saini *et al.*, 2006), this is the first description of iNOS in the nuclei of human cells. Of note, endothelial NOS has been detected in the nuclei of cultured human mast cells (Gilchrist *et al.*, 2004). In those cell lines, endothelial NOS phosphorylation preceded translocation from the cytoplasm to the nucleus. The specific role of nuclear NOS and, implicitly, nuclear NO, has yet to be explored. In melanoma cells, it could be hypothesized that nuclear compartmentalization of iNOS and NO may confer a strategic advantage in terms of efficient modification of DNA and nuclear proteins by NO, in such a way as to promote growth and survival. Although nuclear iNOS was detected in melanocytes as well, these normally quiescent cells are maintained in growth media containing potent mitogens such as cholera toxin and phorbol myristate acetate, providing growth pathway stimulation similar to that seen in tumor cells. It therefore remains to be determined whether nuclear translocation of iNOS is a tumor-specific event. Future research in our laboratory will address this issue as well as the question of iNOS phosphorylation, as has been described for endothelial NOS.

The presence of NF-κB p50 homodimers in the nuclei of melanoma cells is an intriguing finding. This NF-κB subtype may be involved in the competitive transcriptional inhibition of p50/p65, a function frequently attributed to the p50 homodimer (Kang *et al.*, 1992; Plaksin *et al.*, 1993). However, the expression of the transcriptional coactivator Bcl-3 by the melanoma cell lines, and the complexing of Bcl-3 with p50, provides preliminary evidence to suggest that the p50 homodimers may be transcriptionally active. The oncoprotein Bcl-3 is a member of the IκB family of NF-κB-binding proteins. The *BCL3* gene, located on chromosome 19, is translocated intact and is activated in cases of t(14;19)(q32.3;q13.2) B-cell chronic lymphocytic leukemia (Ohno *et al.*, 1990). Bcl-3 has additionally been implicated in the pathogenesis of nasopharyngeal carcinoma, breast cancer, and a variety of lymphomas, all cases giving evidence for coactivation of NF-κB p50 by Bcl-3 as the transforming event (Fujita *et al.*, 1993; Watanabe *et al.*, 1997; Cogswell *et al.*, 2000; Thornburg *et al.*, 2003; Mathas *et al.*, 2005). Bcl-3 expression and its interaction with NF-κB p50 have not been previously reported in melanoma. It is interesting to speculate that Bcl-3/p50 complexes may be important in the transcription of melanoma-promoting genes, such as *INOS*.

In summary, we have further defined the pathway leading from the constitutively active melanoma MAPK pathway to iNOS expression by demonstrating that NF-κB is an important intermediary protein. In the process, we have made the previously unreported observations of the nuclear localization of iNOS and of Bcl-3/NF-κB p50 complexes in cultured melanoma cells. These findings will stimulate further exploration of these regulatory molecules and pathways, always with the ultimate goal of identifying targets for future therapy.

MATERIALS AND METHODS

Cell lines

Dr Robert Kerbel (Sunnybrook Health Science Center, Toronto, ON, Canada) provided the human melanoma cell lines WM793 and

WM35, Dr David Menter (MD Anderson Cancer Center) provided the human metastatic melanoma MeWo cell line, and Dr Ann Richmond (Vanderbilt University School of Medicine, Nashville, TN) provided the HS294T melanoma cell line. BJ fibroblasts were purchased from the ATCC (Manassas, VA). Cells were grown in p line RPMI 1640 medium, supplemented with 10% fetal bovine serum. Primary melanocytes FMC7C and FMC14C were derived from neonatal foreskin and were maintained in MCDB-153 media (Sigma, St Louis, MO), supplemented with 1% fetal bovine serum, 10 ng ml^{-1} phorbal-12-myristate-13-acetate (Calbiochem, San Diego, CA), 1 ng ml^{-1} basic fibroblast growth factor (Invitrogen, Carlsbad, CA), 5 μg ml^{-1} transferrin (Sigma), 10 nM cholera toxin (Calbiochem), 0.1 mM 3-isobutyl-1-methylxanthine (Calbiochem), 30 μg ml^{-1} bovine pituitary extract (Sigma), and 5 μg ml^{-1} insulin (Sigma). All cells were maintained at 37 °C with 5% CO_2.

Reagents and antibodies

NF-κB activation inhibitor and IKK-2 inhibitor V were purchased from Calbiochem and U0126 from Cell Signaling Technology (Beverly, MA). Protein A agarose was obtained from Sigma. Antibodies to ERK, pERK, IκBα, and phosphorylated IκBα were purchased from Cell Signaling Technology. Antibodies to histone, iNOS, and NF-κB p50 and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti Bcl-3 antibody was purchased from Abcam (Cambridge, MA).

Whole-cell extracts and western blotting

Cells were washed with cold phosphate-buffered saline (PBS) and harvested into PBS with 1 mM phenylmethylsulfonyl fluoride. Cell pellets were then treated with lysis buffer (140 mM NaCl, 25 mM Tris-HCl (pH 7.4) and 1% NP-40) with freshly added protease inhibitor cocktail (BD Biosciences, San Jose, CA). The supernatants were then collected after rigorous agitation and protein concentration was measured. SDS–PAGE separated the proteins that were transferred to a nitrocellulose membrane and blocked for 1 hour in 5% nonfat milk in PBS. Primary antibody was diluted in 5% nonfat dry milk/PBS/0.1% Tween and incubated overnight at 4 °C, followed by 45 minutes incubation with horseradish peroxidase-labeled secondary antibody, again diluted in 5% nonfat dry milk/PBS/0.1% Tween. Membrane development was achieved with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Nuclear extracts

The two extraction buffers used, A and B, were composed of the following reagents: buffer A: 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM NaF, 1 mM Na_3VO_4; buffer B: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM Na_3VO_4. Cells were washed with cold PBS and harvested into PBS/1 mM phenylmethylsulfonyl fluoride. Cells were then treated with cold buffer A, followed by the addition of protease inhibitor cocktail 1:50. After the addition of 10% NP-40 and centrifugation at 3,500 *g*, the cytoplasmic fraction was collected. The remaining pellet was washed three times with buffer A, followed by the addition of buffer B and 1:50 protease cocktail inhibitor. After final centrifugation at 16,000 *g*, the nuclear fraction was collected.

Indirect Immunofluorescence

Cells grown on chamber slides were fixed with 2% paraformaldehyde on ice (30 minutes), blocked with 5% serum in PBS (30 minutes room temperature), and incubated with primary antibody diluted in blocking solution (2 hours, 4 °C). This was followed by incubation with fluorescein isothiocyanate-labeled secondary antibody (1 hour, room temperature). Staining was observed and imaged with a Nikon Eclipse TE 2000-U microscope equipped with a Nikon digital DXM 1200F camera.

Electrophoretic mobility shift assay

Nuclear protein extracts for EMSA were prepared as described above. The NF-κB, AP2, and SP1 target oligonucleotides and gel shift binding buffer were purchased from Promega (Madison, WI). Binding reactions were performed with 4 μg of nuclear extract and 1.75 pmol of NF-κB target oligonucleotide (unlabeled or end-labeled with ^{32}P). AP2 or SP1 target DNA sequences were used as nonspecific competitor oligonucleotides. The sequence of the double-stranded NF-κB target oligonucleotide is:

5′-AGTTGAGGGGACTTTCCCAGGC-3′

3′-TCAACTCCCCTGAAAGGGTCCG-5′

DNA–protein complexes were resolved on a 6% nondenaturing gel. The gel was dried and exposed to film at −70 °C. For supershift experiments, 2–4 μg of nuclear extracts were incubated with antibodies to the different subunits of NF-κB for 20 minutes prior to the addition of the radiolabeled probe.

Immunoprecipitation

Whole-cell lysates were prepared as described above. A mixture of protein A agarose, ice-cold PBS, and 1 μg of anti-Bcl-3 or rabbit IgG was tumble incubated overnight at 4 °C. Whole-cell extracts were precleared with protein A agarose for 30 minutes at 4 °C followed by high-speed centrifugation. Precleared lysates were added to the protein A agarose–antibody mixture and incubated for 2 hours at 4 °C. Complexes were washed with wash buffer (0.1% Triton X-100, 50 mM Tris (pH 7.4), 300 mM NaCl, and 5 mM EDTA), resuspended in SDS–PAGE loading buffer, and examined by western blotting.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. BRAF exon 15 sequencing chromatograms for the six cell lines used in the paper.

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